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99th Dahlem Conference on Infection, Inflammation and Chronic Inflammatory Disorders: Induction and control of regulatory T cells in the gastrointestinal tract: consequences for local and peripheral immune responses

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Summary

Regulatory T cells play a crucial role in normal gut homeostasis, as well as during infection with microbial or parasitic pathogens. Prior to infection, interactions with the commensal microflora are essential to differentiation of a healthy steady-state level of immunoregulation, mediated through both Toll-like receptor-dependent and -independent pathways. The ingress of pathogenic organisms may, according to the context, promote or reverse the regulatory environment, with onward consequences for inflammation in both the intestinal and extra-intestinal settings. Appropriate regulation of gut immunity thus depends upon a complex three-way interplay between host cells, commensals and pathogens, and can exert a major impact on systemic responses including allergy and autoimmunity.

Keywords: Colitis, helminths, inflammatory bowel disease, microbiome, protozoa

Introduction

In the gastrointestinal (GI) tract, the immune system is faced with the most demanding of all decision-making, with little room for error. It is imperative at all times to discriminate between, and respond correctly to, beneficial symbionts, harmless food antigens and potential pathogens [1]. There is increasing appreciation that regulatory T cells (T_{regs}) play a prominent and essential role in maintaining appropriate responsiveness in the gut [2,3], actively enforcing homeostasis and preventing untoward immune responses occurring. While stimulated by specific antigens, of both self and non-self origin, T_{regs} can transcend antigen specificity, mediating bystander suppression in a manner likely to modify systemic immune status as suggested by the 'hygiene hypothesis'.

The steady-state: commensals, no infection

Recent studies have changed our perspective of commensal microbes from benign but inert passengers to active participants in both the postnatal development of mucosal immunity and in its long-term steady-state function. Germ-free mice show extensive deficiencies in intestinal immune system development, with reduced lymphoid tissue and fewer lymphocytes [4]. The $CD4^+$ T cell population is diminished, affecting T helper type 1 (Th1) cells disproportionately although, remarkably, T_{reg} frequencies are maintained or increased in germ-free mice. These and other data have established that defined components of the gut flora can play a major role in intestinal homeostasis, including protection against gut injury and mediating oral tolerance against dietary antigens.

In mice which acquire a conventional microbiome, the immune system develops normally while maintaining a continuing dialogue with the commensal population. Here, one of the dominant roles of T_{regs} is to prevent exuberant responses against gut flora, with which the intestinal tract is in intimate contact. Nevertheless, how commensals communicate with cells to ensure immune homeostasis is still unclear. One critical factor in this interaction at the molecular level is the host Toll-like receptor (TLR) system, as demonstrated by spontaneous colitis in TLR-5-deficient mice [5]. Where colitis is induced experimentally (e.g. by dextran sulphate administration), the absence of TLR signalling then results in greatly aggravated pathology, again indicating that TLR-mediated recognition of commensal molecules contributes to dampening immune reactivity [6]. The requirement for TLR signalling in induction of oral tolerance to

dietary antigens [7] also speaks to the bimodal participation of the TLR system in both stimulatory and regulatory arms of the immune response.

Recent evidence suggests that TLR signalling can impact T_{reg} homeostasis and that T_{regs} themselves express TLRs selectively. Hence, interaction with certain ligands, such as those binding TLR-2, can favour T_{reg} expansion both *in vitro* and *in vivo* [8], while TLR-9-mediated recognition of DNA from gut flora is an essential step in ensuring that effector T cells are able, when appropriate, to overcome T_{reg} inhibition and mount an immune response [9].

Alteration of the structural integrity of TLR signalling components is often associated with profound clinical outcome and susceptibility to various infections or autoimmune disorders. During conditions of floral translocation, peripheral TLR-9 signalling is a crucial mediator of polymicrobial sepsis. Moreover, in other conditions in which bacterial translocation occurs [for example, during irradiation and human immunodefiency virus (HIV) infection] peripheral TLR-4 signals enhance the activation status of both $CD4^+$ and $CD8^+$ T cells [10]. However, under most circumstances the tissues of the GI tract are exposed constantly to TLR ligands harboured by the commensal gut flora.

Mice deficient in TLR-9 display increased frequencies of T_{regs} within intestinal effector sites and reduced levels of constitutive interleukin (IL)-17- and interferon (IFN)-γ-producing effector T cells [9]. Complementing this, lamina propria dendritic cells (DCs) lacking exposure to gut flora DNA, induce T_{reg} conversion *in vitro*. Furthermore, T_{reg} *versus* effector T cell disequilibrium in TLR-$9^{-/-}$ mice restricts immune responses to oral infection with the pathogen *Encephalitozoon cuniculi*. Impaired intestinal immune responses were recapitulated in mice treated with antibiotics and were reversible after reconstitution with gut flora DNA [9]. Thus, signals derived from the gut flora act as adjuvants of immune responses for priming intestinal responses against oral pathogens via modulation of the equilibrium between T_{reg} and effector T cells.

Intestinal epithelial cell (IEC) expression of TLRs has also proved to be important in maintaining the homeostatic host–microbiome relationship, and to involve unexpected subtleties. For example, TLR-9 is expressed on both the apical (luminal-facing) and basolateral surfaces of the epithelial cell layer, but only basolateral ligation triggers an inflammatory signal, while apical binding is inhibitory [11]. The capacity of IECs to control immune responsiveness extends to the production of thymic stromal lymphopoietin (TSLP) and IL-25, influencing the Th phenotype balance in a manner which can make or break effective immunity [12].

Not all commensals are equal

The structure and composition of the gut flora reflect natural selection at both the microbial and host levels, and show perturbations in GI dysfunction. For example, modified gut floral composition is found in inflammatory bowel disease (IBD) patients [13]. Furthermore, the presence of certain bacteria can aggravate small intestinal immunopathology following oral infection. There can be exquisite specificity in the effects of individual commensal species; for example, within the genus *Lactobacillus* different species are associated with differing *in vivo* allergic status in infants [14] and contrasting capacity to induce regulatory cytokines *in vitro* [15]. Moreover, even different strains or mutants of particular *Lactobacillus* species stimulated very different immunological outcomes in mice [16,17].

Recent evidence demonstrates that colonization of germ-free mice with complex microbiota orchestrated a broad spectrum of Th1, Th17 and T_{reg} responses. Whereas most tested individual bacteria failed to stimulate intestinal T cell responses efficiently, a restricted number of individual bacteria can control the tonicity of the gut immune system [18]. The key commensal organisms in immune system development have been identified very recently as segmented filamentous bacteria [18,19]. A further reflection of how the make-up of the intestinal flora can impact upon systemic responses is found in studies of non-obese diabetic (NOD) mice, which succumb spontaneously to type 1 diabetes (T1D); it has been known for some time that higher microbial exposure militates against development of this autoimmune disease [20], but it was shown recently not only that conventionally housed myeloid differentiation primary response gene 88 $(MyD88)^{-/-}$ mice are resistant to T1D, but that resistance to disease is due to the distinct microbial combination with which they are colonized. Hence, $MyD88^{-/-}$ mice develop T1D under germ-free conditions, while wild-type mice given the microbial population from $MyD88^{-/-}$ animals had reduced susceptibility to disease [21].

It is tempting to speculate that alteration of T_{reg} homeostasis mediated by TLR signalling, either because of genetic polymorphism or because of changes in gut flora composition, could also have consequences on development of gut inflammatory disorders. Indeed, gut flora bacteria are not equal in their capacity to stimulate TLR-9 and do so with various levels of efficiency that correlate with the frequency of cytosine–guanine dinucleotides. Thus, control of the T_{reg} ratio and effector T cell function in the GI tract is likely to be regulated differentially by specific gut flora species. An illustration of how the presence of defined bacterial species can influence the outcome of an infection comes from the observation that mice fed *Bifidobacterium infantis* are protected from the pathogenic effect and translocation of *Salmonella* [22]. Activation of T_{regs} by the probiotic microorganism contributed to this protective effect.

Can commensals regulate pathology?

The proposition that certain commensal species may act in a counterinflammatory manner has led to extensive investigation of potential probiotic regulation of immunopathology.

Promising results have been obtained with probiotics in the treatment of human inflammatory diseases of the intestine and in the prevention and treatment of atopic eczema in neonates and infants, but mechanism(s) of action remain to be elucidated [23]. In mice, probiotic *Lactobacillus* and/or *Bifidobacterium* treatment suppressed trinitrobenzene sulphonic acid (TNBS)-induced colitis [24,25], as well as allergy, while raising transforming growth factor (TGF)-β production [26] and stimulating T_{regs} able to transfer down-modulation of allergy [27]. There is also good evidence of probiotic modulation of DCs towards a proregulatory function [15,28]. Of course, not all commensals are down-regulatory, and some (like *Helicobacter hepaticus*) may be pathogenic in some settings, yet induce T_{regs} in others [29]. Furthermore, there can be significant interactions between pathogens, as in the example of intestinal bacteria aggravating the immunopathology caused by *Toxoplasma* infection [30]. In the latter setting, there is reduced floral complexity, either because of relative loss of more 'regulatory' strains or simply as a broad reflection of an altered homeostasis accompanying pathogenesis.

One consequence of the immune system's reliance on microflora for optimal immunoregulation is that antibiotic therapies may result in unintended activation of immune effector mechanisms. In model systems, antibiotic treatment renders mice more susceptible to induction of food allergy [7] as well as allergic airway inflammation [31]. For the human population, antibiotics are seen as major modifiers of beneficial human–microbe interactions [32] superimposed upon alterations caused by other exogenous factors including urbanization, global travel and dietary changes [33]. The acute effects of antibiotic treatment on the native gut microbiota range from self-limiting diarrhoea to life-threatening pseudomembranous colitis induced by bacteria filling the niche provided by the reduction in bacterial diversity [34]. The long-term consequences of such perturbations for the human–microbial symbiosis are more difficult to discern, but chronic conditions such as asthma and atopic disease have been associated with childhood antibiotic use and an altered intestinal microbiota [35–37].

Because many chemical transformations in the gut are mediated by specific microbial populations, with implications for, among others, cancer and obesity, changes in the composition of the gut microbiota could have important but undiscovered health effects. In this regard, ciprofloxacin treatment of healthy volunteers influenced the abundance of about a third of the bacterial taxa in the gut, decreasing the taxonomic richness, diversity and evenness of the community. However, the magnitude of this effect varied among individuals, and some taxa showed interindividual variation in the response to ciprofloxacin. In each individual, the taxonomic composition of the community closely resembled its pretreatment state by 4 weeks after the end of treatment, but several taxa failed to recover within 6 months [38].

The production of active anti-inflammatory mediators by particular commensal species (reviewed in [39]) provides a mechanistic framework for microbial regulation of pathology in the GI tract. One of the most striking examples is the zwitterionic polysaccharide A of *Bacterioides fragilis*, which restores $CD4^+$ T cell numbers in germ-free mice [40] and protects mice against *Helicobacter*-induced colitis with the induction of IL-10-producing Tr1 cells [41]. Hence, immunoregulation may revolve around highly specific host–microbial molecular interactions, presumably reflecting a long and intimate co-evolution of the symbiotic relationship.

De novo induction of T_{regs} in the GI tract

The vitamin A metabolite, retinoic acid (RA), plays a major role in the GI tract, via its capacity to enhance the TGF-β-mediated generation of forkhead box P3 ($FoxP3^+$) T_{regs} from naive T cells by gut DCs [42]. Reciprocally, RA can inhibit the generation of Th17 cells [43], suggesting that it may play an important role in maintaining the balance between effector and regulatory populations in the GI tract. Several populations of mucosal APC can induce T_{regs} via RA, although only the CD103 subset is equipped with the enzymatic machinery to generate RA.

Retinoic acid can also imprint gut homing molecules on various populations of lymphocytes. Defined microenvironments may have evolved self-contained strategies in which local mediators (such as RA) can imprint homing properties while also favouring the induction or function of T_{regs}. It is therefore tempting to speculate that a link between homing and regulatory function induction may represent a more general mechanism. Such a strategy could allow the constant generation and migration of T_{regs} to defined compartments. These T_{regs} would be expected to have the prerequisite antigen specificities (e.g. persistent microorganisms, flora antigens), status of activation and survival requirement that allow them to regulate a defined microenvironment.

Although the capacity of gut-associated lymphoid tissue (GALT) DCs or macrophages to imprint gut-homing receptors and induce $FoxP3^+$ T_{regs} is associated with their capacity to release RA, it remains unclear if these cells are the main producers of this metabolite in the gut. Synthesis of RA from stored or dietary retinol depends on the direct expression of the appropriate enzymes by GALT DCs. Certainly, DCs from Peyer's patches and mesenteric lymph nodes (MLNs) express Aldh1a1 and Aldh1a2, respectively, and $CD103^+$ DCs from the lamina propria express a large array of this family of enzymes; moreover, Peyer's patch and MLN DCs can convert retinol directly to RA in culture. However, other cells, including IELs, can express enzymes associated with vitamin A metabolism, suggesting that DCs may also acquire retinoic acid from other sources and store it. A recent study demonstrated that monocyte-derived DCs pretreated with RA can acquire several attributes characteristic of mucosal DCs,

such as secretion of TGF-β and IL-6, and the capacity to augment mucosal homing receptor expression and IgA responses in lymphocytes [44]. In this particular study, these gut-derived features acquired by DCs were associated with the capacity of DCs to become carriers and not producers of RA. The precise factors that govern the activation of some of these enzymes as well as how inflammation or infections modify the metabolism of vitamin A remain to be explored. Importantly, how commensals contribute to the expression of these enzymes and metabolism of vitamin A remains unknown. Another important question is the timing necessary for DCs migrating in the GALT to acquire RA from epithelial cells and how these processes can be modified during infection. How RA contributes to oral tolerance, and at the same time protective immunity in the GI tract, also remains to be addressed. One possibility is that RA favours the induction of T_{regs} in the absence of secondary signals but enhances effector responses following exposure to inflammatory mediators.

T_{reg} populations require not only appropriate conditions for their induction, but also for their upkeep, particularly when confronted with an inflammatory environment. Very recently it has been shown that, in the gut, myeloid cell-derived IL-10 plays a crucial role in maintaining functional T_{reg} activity by stimulating IL-10R directly on FoxP3$^+$ T_{regs} and allowing them to play a fully protective role in the prevention of colitis [45]. Thus, in the absence of either innate IL-10 production, or IL-10R on T_{regs}, these cells lose the ability to block colitogenic effector T cells from causing inflammatory disease, and indeed succumb themselves to the inflammatory process by switching to the production of IFN-γ [45]. Hence, IL-10 is important for the maintenance of T_{reg} activity and can be pivotal at the tipping-point between regulation and inflammation.

Systemic effects of GI regulation

The regulation of T_{reg} activity between the gut and the periphery is also of special interest, as IBD in humans may affect extraintestinal organs in up to 36% of cases [46]. IBD-related extraintestinal disorders are not specific to IBD. They can be classified into reactive manifestations dependent directly upon intestinal disease. The often co-existing presentation in the same patient points towards common underlying pathomechanisms that may involve enteric flora activating the immune system to turn against bacterial antigens and, based on cross-reactivity, against intestinal antigens and antigens in extraintestinal organs ('molecular mimicry').

A separate subset of IBD patients shows an increased frequency of other common autoimmune diseases that manifest mainly independently of the bowel disease. This may thus reflect susceptibility to autoimmunity in general.

The complex relationship between intestinal and extraintestinal manifestations in IBD is also reflected by the complex multi-genetic control reported in animal models of IBD; genetic loci regulating intestinal and extra-intestinal manifestations are largely but not exclusively different [47].

Parasite infection: pro- or counter-regulatory?

The appearance of GI parasites is a major challenge to the discriminatory powers of the immune system, and one which in evolutionary time has been played out countless times. The host rarely fails to respond to the infection, but the outcome in terms of response mode (Th1, Th2, Th17) and the degree of T_{reg} activation varies markedly according the pathogen in question. In recent years, good experimental data has been provided to show that host regulatory pathways are activated by certain GI parasites in particular helminths. For example, the duodenal-dwelling nematode *Heligmosomoides polygyrus* can inhibit gut inflammation in the mouse associated with *Helicobacter* colitis [48], genetic IL-10 deficiency [49] or peanut allergy [50]; the same parasite stimulates T_{reg} expansion and induction *in vivo* and *in vitro* [51–53]. In *Trichuris muris* infections of the colon, T_{regs} are required to minimize intestinal pathology and the parasite strain able to survive longest in the mouse is associated with the largest numerical expansion in T_{regs} [54].

Although data from human helminth infections are not so definitive, new and remarkable evidence has been provided for the presence of GI helminth-associated T_{regs}. A cohort of multiple sclerosis patients were found to have acquired gut helminth infections while under longitudinal monitoring in the clinic; infected individuals showed a dramatically lower rate of relapse, with milder clinical scores, than case–controlled uninfected patients. Infected subjects showed higher correlates of T_{reg} activity and lower inflammatory cytokine production on autoantigen stimulation, linking the helminth infection with expanded T_{reg} activity and improved clinical outcome [55].

Studies to date have not been defined whether the T_{reg} subsets stimulated by GI helminths are natural or induced, or if there are parasite-specific T_{reg} populations among them. In addition, the relative importance of Tr1 (non-FoxP3-expressing, IL-10-producing) regulatory cells is brought into question by the dispensible nature of IL-10 for many helminth-associated regulatory effects (for example [56]). By contrast, new data are clearly demonstrating an inherent capacity to promote induced T_{reg} development and function in the case of *H. polygyrus* secretions which drive *de novo* expression of FoxP3 in naive peripheral T cells.

The distinction between T_{regs} and inducible regulatory T cells *in vivo* is not always clear, particularly in highly inflammatory settings. Moreover, T_{regs} may be able to influence the emergence or function of one another. This notion was suggested recently in a model of *Aspergillus conidia* infection in mice. In this model, control of allergic immunopathology induced by the fungus required the sequential activity of various populations of T_{regs} [57]. This sequential role for

various populations of T_{regs} may not be an exception but rather the rule, as most infections proceed through various stages and therefore require various layers of regulation.

The host, on the other hand, has many mechanisms which may uphold or restore responsiveness in a counter-regulatory fashion. For example, upon infectious challenge the gut-resident APC are likely to be replaced by inflammatory cells that have not been conditioned by the gut environment [58]. Previous reports examining both gut and lung inflammation support the idea that restricted or defective T_{reg} conversion can enhance immunopathology [59]. Such limitations of conversion during inflammation raise the possibility that exposure to antigen at a time of acute infection may impair the acquisition of tolerance against commensals that could, in turn, contribute further to the pathological process. Whatever the mix of factors at play, it is clear that regulation by pathogens is a dynamic process and, under the right circumstances, host immunity can reassert itself to overcome the infection.

Systemic consequences and long-term effects of GI infections

If changes in the commensal population within the GI tract impact upon systemic immune responses, as discussed above, then it is not surprising to find that parasitic infections in the same milieu can also exert substantial systemic effects. The influence of infection on 'bystander' responses, particularly where mediated through various regulatory cell populations, provides a mechanistic explanation of the more general 'hygiene hypothesis' concept that increasing rates of allergy and asthma in western countries could be the consequence of reduced infectious stresses during early childhood [60].

Experimental work has lent strong support for this hypothesis. For example, during GI infection, helminth-driven T_{reg} suppression of effector function protects against subsequent airway inflammation [56]. Similar infections change responses to blood-stage malaria [61] and interfere with vaccinations [62,63]. Evidence for bystander suppression in human GI helminth infection is also accumulating, with lower allergy rates in infected children [64,65], and lower inflammatory responses to autoantigen in the multiple sclerosis study mentioned above [55]. Indeed, helminth therapy is being trialled as a potential strategy to ameliorate intestinal inflammation in Crohn's disease and ulcerative colitis [66]. Notably, other suppressive cell types are observed in these infections, including 'regulatory B cells' and alternatively activated macrophages, although the interdependence and sequence of activation of these other regulatory components have yet to be discerned [67].

Pathogens may therefore have evolved to exploit, and even imitate, our symbiotic relationship with gut flora. As described above, probiotic microorganisms have beneficial effects in the treatment of inflammatory bowel diseases through the induction of T_{reg} populations, and evidence is now emerging that some helminths can act similarly. As with commensal microbes, different helminths exert very different immunological effects and some appear to be less adept in anti-inflammatory action than others, as ongoing research is now establishing.

The presence of symbiotic and pathogenic microorganisms in the gut or other peripheral tissues could lead to the maintenance of a pool of activated T_{regs} (both natural and inducible) that would maintain host immune homeostasis and enhance the threshold required for immune activation and induction of an immune response. The benefit of such deactivation is to decrease the instances of aberrant immune responses, such as allergic and autoimmune disorders. Pathogenic microorganisms may also have evolved to express antigens that cross-react with gut flora antigens. In infections, the removal or modification of the gut flora is associated with a modification of the phenotype of the host responses. Therefore, some microorganisms may hijack T_{regs} that are induced or activated in the gut to limit pathogenic responses against gut flora to ensure their own survival.

Over time, established GI infections may create a new homeostatic set point, in which reactivity to the chronic pathogen is minimized, with wider implications for responsiveness to self-antigens and allergens which may not be altogether detrimental. At this point, it remains unclear to what extent any recalibration of host immunity is induced purely by the pathogen, or by perturbation of the commensal population, or is a result of endogenous controls within the immune system itself. On the basis of both human and experimental studies discussed above, it seems likely that all three components play an essential role in reaching a stable and nonpathogenic steady state for the longer term.

Disclosure

None.

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